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Defining the affinity and receptor sub-type selectivity of four classes of endothelin antagonists in clinically relevant human cardiovascular tissues

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ABSTRACT

Aims: We have compared the endothelin receptor subtype affinity (K_D) and selectivity of four structural classes of antagonists (peptide, sulphonamide-based, carboxylic acid-based, myceric acid-based) in human cardiovascular tissues to determine whether these are predicted by values reported for human cloned receptors. Additionally, affinities (K_B) for these antagonists, determined in ET-1-mediated vasoconstriction assays in human blood vessels, were used to identify discrepancies between K_B and K_D determined in the same tissues.
Main methods: Competition binding experiments were carried out in sections of human left ventricle, coronary artery and homogenates of saphenous vein to determine K_D values for structurally different ET_A-selective (FR139317, BMS 182874, S97-139, sitaxentan, ambrisentan) and mixed (PD142893, Ro462005, bosentan, L-749329, SB209670) antagonists. Schild-derived values of antagonist affinity were obtained in vascular functional studies.
Key findings: When compared with previously reported data in human cloned endothelin receptors, those antagonists reported to be ET_A-selective exhibited even greater ET_A selectivity in human ventricle (BMS 182874, sitaxentan, ambrisentan) that expressed both receptor subtypes. Those antagonists reported to have <100 fold selectivity in cloned receptors (PD142893, Ro-462005, bosentan, SB209670, L-749329) did not distinguish between receptor subtypes in human left ventricle. For antagonists where we determined affinity in vascular functional and binding assays (Ro462005, bosentan, BMS 182874, L-749329, SB209670) there was no correlation between the degree of discrepancy in K_B and K_D and structural class.
Significance: For an antagonist to retain ET_A-selectivity *in vivo* it may be necessary to identify those compounds that have at least 1000 fold ET_A:ET_B selectivity in *in vitro* assays.

Introduction

The endothelins (ETs) are a family of three endogenous twenty-one amino acid peptides, ET-1, ET-2 and ET-3 (Inoue et al., 1989; Yanagisawa et al., 1988) that mediate their action *via* two G-protein coupled receptors, ET_A (Arai et al., 1990; Hosoda et al., 1992) and ET_B (Sakurai et al., 1990). In the human vasculature ET-1, released from endothelial cells acts on the ET_A receptors that predominate on the underlying smooth muscle cells throughout the human cardiovascular system and mediate vasoconstriction. In some vessels a small population of ET_B receptors (usually <15%) are also present and these may also mediate constriction (Davenport and Maguire, 1994; Maguire and Davenport, 1995) although infusion of the ET_A selective peptide antagonist, BQ-123, into healthy volunteers caused progressive vasodilatation (Haynes and Webb, 1994), consistent with ET-1 being continuously released to cause vasoconstriction *via* this subtype. In contrast infusion of an ET_B selective antagonist, BQ788, caused systemic vasoconstriction, showing that the main consequence of activation of endothelial ET_B receptors by tonically secreted ET-1 was release of nitric oxide (Love et al., 2000). The ET_B receptor also functions as a clearing receptor, to internalise the ligand-receptor complex and remove ET-1 from the circulation (Fukuroda et al., 1994; Gasic et al., 1992; Plumpton et al., 1996). As a result the plasma half life of ET-1 is comparatively short. ET_B receptors are expressed at particularly high densities in human lung, kidney and liver (Davenport and Russell, 2001; Gariepy et al., 2000; Kuc et al., 1995).

Overproduction of ET in pathophysiological conditions may lead to vasospasm, particularly in conditions of endothelial dysfunction where there is loss of counter regulatory vasodilators such as nitric oxide, prostacyclin and endothelium derived hyperpolarising factor. Endothelin is thought to contribute to processes associated with vascular remodelling such as smooth muscle cell proliferation, fibrosis and inflammation. Antagonists that block the endothelin receptors either selectively or non-selectively were originally developed for the treatment of heart failure. Clinical trial data were disappointing in this

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condition, however subsequently two antagonists, the non-selective compound bosentan (Tracleer) (Rubin et al., 2002) and the ET_A antagonist ambrisentan (Letairis, Volibris) (Vatter and Seifert, 2006) have been introduced for treatment of pulmonary arterial hypertension (PAH). The more ET_A selective antagonist sitaxentan (Thelin) (Benza et al., 2007; Galie et al., 2009) was recently withdrawn voluntarily from the market. Although endothelin receptor antagonists have become established as having therapeutic benefit in PAH (Liu et al., 2009; McLaughlin et al., 2009), the relative merits of mixed ET_A/ET_B or selective ET_A antagonism continue to be debated (Davie et al., 2009; Dupuis and Hoeper, 2008; Vachiery and Davenport, 2009). Many of the detrimental actions of ET-1 are thought to result from the activation of the ET_A receptor that predominates in human vascular smooth muscle and mediates vasoconstriction. Beneficial actions result from stimulation of the ET_B receptor and include vasodilatation, clearing ET from the circulation, diuresis and natriuresis (Davenport and Maguire, 2006). New clinical indications for ET antagonists are emerging, including chronic kidney disease (Karet and Davenport, 1996; Goddard et al., 2004; Karet et al., 1993; Kohan, 1997, 2010; Longaretti and Benigni, 2009; Neuhofer and Pittrow, 2009) autoimmune disorders of the vasculature such as scleroderma (Abraham and Dashwood, 2008) and remarkably in cancer (Bagnato et al., 2008; Bhalla et al., 2009; Growcott, 2009). In order to determine the relative benefit of mixed ET_A/ET_B compared to ET_A-selective blockade in these conditions it is important to quantify the endothelin receptor selectivity of antagonists. For the most part this has been achieved using human cloned receptors or cells/animal tissues that endogenously express only (or predominantly) one or other receptor subtype. We were interested to know how closely these data predict the affinity and selectivity of antagonists for human receptors expressed in clinically relevant human tissues. We have previously determined affinity of a number of antagonists in radioligand binding assays using human heart or blood vessels and carried out functional experiments on some of these compounds to determine their ability to antagonise ET-1 mediated vasoconstriction in human artery and vein. We have now extended these studies to provide additional data for antagonists from each of four different chemical classes (Palmer, 2009): the peptide PD142893; the sulphonamides Ro462005, bosentan, BMS 182874, L-749329 and sitaxentan; the carboxylic acids SB209670 and ambrisentan and the myceric acid S97-139. Our aim was to compare the affinity of these ET receptor antagonists obtained in our pharmacological binding and functional assays using human tissue together with values that we have previously reported for other antagonists in these four classes to values reported in the literature for the same compounds using cloned human receptor systems.

Methods

Human tissue

Anonymised human cardiovascular tissue samples were used in this study with local ethical approval (REC 05/Q0104/142). The samples were obtained from Papworth Hospital Research Tissue Bank (Cambridgeshire 1 Research Ethics Committee reference 08/H0304/56, samples collected with written informed patient consent). Unless otherwise stated n-values refer to the number of patients from whom tissue was obtained.

Competition binding assays in human cardiovascular tissues

The binding experiments were performed in the presence of bovine serum albumin (BSA) to more closely reflect *in vivo* conditions, as some of these compounds are known to show appreciable binding to plasma proteins (Wu-Wong et al., 1996). Competition binding experiments were carried out as previously described (Davenport and Kuc, 2005; Davenport et al., 1995; Maguire et al., 1996). Briefly, sections of human heart (10 μm), coronary artery (10 μm) or homogenates of saphenous vein (Maguire et al., 1996) were pre-incubated in HEPES buffer and then with [^{125}I]ET-1 (0.1 nM) for 2 h at room temperature in the presence of increasing concentrations of antagonists (2 pM–100 μM). Non-specific binding was determined in the presence of 1 μM of the unlabelled ligand. Affinities (K_D the equilibrium dissociation constant) of antagonists for ET_A and ET_B receptors were determined using the non-linear curve fitting programme KELL (Biosoft, http://Biosoft.com). For each antagonist data were obtained in tissue from at least three individuals.

Human vasoconstriction functional assay in human artery and vein

Experiments were carried out as previously described (Maguire, 2002). Briefly, 4 mm rings of endothelium-denuded human coronary artery or saphenous vein were set up for isometric force recordings. Cumulative CRCs were constructed to ET-1 (10^{-10}–10^{-6} M) in the absence (control) and presence of three or four concentrations of each antagonist. Blood vessel preparations were incubated with antagonists for 30–40 min prior to addition of ET-1. Experiments were terminated by the addition of 100 mM KCl to determine the maximum possible vasoconstrictor response for each tissue and ET-1 responses were expressed as a % of this terminal KCl response. Data were analysed using the non-linear iterative curve-fitting programme FigSys (Biosoft) and antagonist affinities (K_B the equilibrium dissociation constant) were determined by Schild analysis (Arunlakshana and Schild, 1959). If not significantly different from one, Schild slopes were constrained to one to determine the value of K_B. For each concentration of antagonist data were obtained in vascular tissues from 3 to 10 individuals.

Materials

ET-1 was from The Peptide Institute (Osaka, Japan). Sitaxentan was a gift from Pfizer Inc., ambrisentan was a gift from Gilead Sciences Inc. Other antagonists were kindly supplied by Parke-Davis. [^{125}I]-ET-1 (2200 Ci/mmol) was from PerkinElmer NEN® Radiochemicals.

Results

Selectivity of endothelin receptor antagonists determined in human left ventricle

Peptide antagonists: In human left ventricle the modified peptide PD142893 exhibited four-fold selectivity for the ET_A receptor (ET_A K_D 0.30 ± 0.03 μM; ET_B K_D 1.17 ± 0.14 μM).

Sulphonamide antagonists: BMS 182874 was found to have high nanomolar affinity for the ET_A receptor (K_D 590 ± 100 nM) but no detectable affinity for the ET_B receptor. Sitaxentan was also highly ET_A selective (ET_A K_D 1.65 ± 0.80 nM; ET_B K_D 327 ± 134 μM) whereas a one site fit was preferred for L-749329 (K_D 303.5 ± 34.3 nM) indicating that this compound could not distinguish between the two receptor subtypes.

Carboxylic acid antagonists: Ambrisentan had high affinity for the ET_A receptor and almost 1000 fold selective for this subtype over the ET_B receptor (ET_A K_D 0.28 ± 0.23 nM; ET_B K_D 0.25 ± 0.05 μM).

Myceric acid antagonists: S97-139 (ET_A K_D 45.3 ± 25 nM; ET_B K_D 47.6 ± 9.9 μM) was 1000 fold selective for the ET_A receptor in human heart.

Affinity of antagonists for the ET_A receptor determined in saphenous vein binding experiments

Peptide antagonists: In human saphenous vein homogenate the ET_A receptor comprises 85% of the total ET receptor population (Davenport et al., 1995). The peptide FR139317 competed with high

affinity for the ET_A receptor (K_D 0.56 ± 0.01 nM) but had no detectable affinity for the low density ET_B receptor.

Sulphonamide antagonists: A one-site fit was preferred for Ro462005 (K_D 0.15 ± 0.01 μM) and bosentan (K_D 32.2 ± 3.2 nM) which competed for 100% of the [^{125}I] ET-1 binding whereas L-749329 (ET_A K_D 66.7 ± 7.5 nM) and BMS 182874 (ET_A K_D 580 ± 40 nM) competed for 85–90% of [^{125}I] ET-1 binding but affinities for the small ET_B population could not be measured.

Carboxylic acid antagonists: SB209670 competed for all of the [^{125}I] ET-1 binding with a K_D of 11.2 ± 1.4 nM.

Affinity of antagonists for the ET_A receptor determined in saphenous vein and coronary artery functional experiments

For all of the antagonists tested, the Schild slopes were not significantly different from one and therefore slopes were constrained to one to obtain values of K_B.

Peptide antagonists: The Schild-derived affinity for antagonism of ET-1 by PD142893 (3, 10 30 μM) in saphenous vein was K_B of 5.96 ± 0.13 (slope = 0.64 ± 0.34, n = 3–6) and in coronary artery (1, 3, 10 μM) K_B was 5.83 ± 0.10 (slope = 0.67 ± 0.22, n = 4–7).

Sulphonamide antagonists: Affinities for Ro462005, bosentan, BMS 182874 and L-749329 were obtained in saphenous vein and coronary artery. In vein Ro462005 (3, 10, 30 μM) had K_B = 5.65 ± 0.07 (slope = 1.05 ± 0.19, n = 4–5) and in artery K_B = 5.87 ± 0.15 (slope = 1.21 ± 0.38, n = 4–5). For bosentan (0.3, 1, 3, 10, 30 μM) values in vein were K_B = 5.80 ± 0.25 (slope = 0.96 ± 0.39, n = 3) and in coronary artery K_B = 5.53 ± 0.17 (slope = 0.91 ± 0.30, n = 3–5). BMS 182874 (1, 3, 10 μM) in saphenous vein K_B = 6.56 ± 0.10 (slope = 0.94 ± 0.32, n = 3–6) and in coronary artery K_B = 6.18 ± 0.09 (slope = 0.94 ± 0.24, n = 4–8). L-749329 (30, 100, 300 nM) had K_B = 8.19 ± 0.17 (slope 0.68 ± 0.44, n = 6–7) in vein and K_B = 7.91 ± 0.09 (slope 1.19 ± 026, n = 4–9) in artery. Affinity for sitaxentan (1, 3, 10 μM) was determined in saphenous vein; K_B = 7.21 ± 0.16 (slope = 1.10 ± 0.50, n = 7–9).

Carboxylic acid antagonists: For SB209670 (0.1, 0.3, 1, 3 μM) in saphenous vein K_B = 7.93 ± 0.19 (slope = 0.79 ± 0.19, n = 3) and similar affinity was obtained in coronary artery (0.1, 0.3, 1 μM) K_B 7.70 ± 0.15 (slope = 0.61 ± 0.35, n = 3–5). In contrast to its subnanomolar affinity determined for the cardiac ET_A receptor in saphenous vein in binding assays ambrisentan (0.3, 3, 10 μM) was a much less effective antagonist of ET-1 vasoconstriction than expected with K_B = 6.76 ± 0.15 (slope = 0.69 ± 0.23, n = 6–7).

Myceric acid antagonists: For S97-139 (10, 30, 100 and 300 nM) in saphenous vein K_B = 7.97 ± 0.16 (slope = 0.7 ± 0.3, n = 3–6) and in coronary artery K_B = 7.61 ± 0.12 (slope = 0.70 ± 0.27, n = 3–4).

Discussion

The aim of our investigation was to address the question of whether values of antagonist affinity and receptor subtype selectivity, obtained from cloned human receptors (Aramori et al., 1993; Breu et al., 1993; Clozel et al., 1994; Doherty et al., 1993; Elliott et al., 1994; Fujimoto et al., 1992; Mihara et al., 1994; Patt et al., 1997; Walsh et al., 1994; Webb et al., 1995; Williams et al., 1993; Wu et al., 1997; Vatter and Seifert, 2006) predict those obtained in human tissues that express both receptor subtypes (Davenport and Russell, 2001) and which are the intended target for endothelin antagonists clinically; specifically the heart (Love et al., 2000; Peter and Davenport, 1995, 1996), lungs (Davie et al., 2009; Dupuis and Hoeper, 2008; Liu et al., 2009; McLaughlin et al., 2009; Vachiery and Davenport, 2009), kidney (Karet and Davenport, 1996; Kuc et al., 1995; Goddard et al., 2004; Karet et al., 1993; Kohan, 1997, 2010; Longaretti and Benigni, 2009; Neuhofer and Pittrow, 2009) and vasculature (Abraham and Dashwood, 2008; Bacon and Davenport, 1996; Davenport and Maguire, 1994, 2006; Davenport et al., 1995; Maguire and Davenport, 1995). We focussed on human heart as ET receptor sub-types are present in the left ventricle in a ratio of about 60% ET_A:40% ET_B which allows for the accurate measurement of affinity constants for antagonists against both receptors in the same experiments (Davenport and Kuc, 2005; Molenaar et al., 1993; Peter and Davenport, 1995, 1996). We also determined affinities (K_D) in competition experiments in human vascular tissues (coronary artery and saphenous vein) (Bacon and Davenport, 1996; Davenport et al., 1995; Maguire et al., 1994) for comparison to their affinities (K_B) determined from the antagonism of ET-1 mediated vasoconstriction in rings of these blood vessels in organ bath experiments (Davenport and Maguire, 1994; Maguire, 2002; Maguire and Davenport, 1995; Maguire et al., 1994, 1996, 1997). We identified a number of antagonists, belonging to different structural classes; peptides, sulphonamides, carboxylic and myceric acids (Palmer, 2009) that included both the reported ET_A selective and mixed receptor antagonists. We have summarised the data reported in the present study together with human data that we have previously reported for antagonists from the four classes and compared these values to those reported in the literature using human cloned receptors (Table 1). From this it is apparent that the degree of selectivity for those antagonists in the peptide, sulphonamide and carboxylic acid classes, reported to be ET_A selective (BQ123, FR139317, sitaxentan, BMS 182874, PD156707, ambrisentan) was markedly increased from ~200–7500 fold in cloned receptor cell systems to ~800–200,000 fold in the human left ventricle binding assay. As expected, those antagonists that had been reported to be mixed antagonists (ET_A selectivity <100, PD142893, Ro462005, bosentan, L-749329, SB209670), we found that these compounds did not distinguish between the two receptors in the human heart (Table 1). The myceric acid antagonists were the only group in which selectivity was as good or better in cloned receptors compared to native receptors. Differences in assay conditions such as receptor over-expression and co-expression with non-physiologically relevant G-proteins may explain some of the observed discrepancies. In some cloned receptor studies different radioligands, [^{125}I]-ET-1 or [^{125}I]-ET-3, were used to label the two subtypes ET_A and ET_B receptors respectively, whereas we used [^{125}I]-ET-1 to label both populations of receptor subtypes in the human left ventricle. Agonist dependence of antagonist affinity has been reported for some endothelin antagonists and may contribute, at least in part, to our observed discrepancies.

If selective blockade of the vascular ET_A receptor is clinically desirable how predictable of functional potency is the affinity of an antagonist determined in a receptor binding assay? To address this question we have carried out additional experiments to measure how well the ET_A affinity of selective and mixed antagonists, determined in binding experiments, reflects their potency as functional antagonists at the ET_A receptor *in vitro*. We carried out Schild analysis of data obtained from the antagonism of ET-1 induced vasoconstriction in human isolated coronary artery and/or saphenous vein (an ET_A response (Davenport and Maguire, 1994; Maguire and Davenport, 1995)) for representative antagonists from each structural group and have summarised these together with the data previously reported for additional antagonists from the four groups in these human tissues (Table 2). The Schild-derived affinity values (ET_A K_B) and the ET_A affinity (K_D) determined in binding experiments in the same vascular tissues were compared and expressed as a K_B/K_D ratio (Table 2). These data indicated that for the peptide antagonists concentrations required to block ET-1 vasoconstriction were 100–2000 fold higher than predicted by their ET_A binding affinity determined in competition binding experiments in the same tissues using [^{125}I] ET-1. For the non-peptide antagonists the degree of K_D to K_B discrepancy was much more variable; ranging from ten fold higher affinity in functional compared to binding assays (L-749329) to 1000 fold lower affinity determined in functional compared to binding assays (bosentan). Overall there was no apparent correlation between structural class and the discrepancy in K_B and K_D values determined in the two assay systems. Therefore, if these data are to be extrapolated to the clinical setting for some antagonists, such as bosentan, the concentration required to

Table 1
Summary of endothelin receptor subtype affinity and selectivity of antagonists reported for human left ventricle compared to values reported for human cloned receptors.

Antagonist	Cloned receptors[a]			Human left ventricle		
	K_i ET_A	K_i ET_B	ET_A selectivity	K_D ET_A	K_D ET_B	ET_A selectivity
Peptides						
BQ123	17 nM	11.1 μM	653[1]	0.73 ± 0.22 nM	24.3 ± 2.0 μM	33,288[2]
FR139317	1 nM	7.3 μM	7300[3]	1.20 ± 0.28 nM	287 ± 93 μM	239,167[4]
PD151242			ND	7.21 ± 2.80 nM	104 ± 23 μM	14,424[5]
PD142893	31 nM[a,b]	54 nM[a,b]	1.7[6]	**0.30 ± 0.03 μM**	**1.17 ± 0.14 μM**	**4**
Sulphonamides						
Ro-462005	360 nM[b]	530 nM[b]	1.5[7]	One site fit 50.3 ± 9.5 μM[4]		Non-selective[4]
Bosentan	4.7 nM	95 nM	20[8]	One site fit 77.6 ± 7.9 nM[4]		Non-selective[4]
Sitaxentan	1.4 nM[b]	9.8 μM[b]	7000[9]	**1.65 ± 0.80 nM**	**327 ± 134 μM**	**198,182**
BMS 182874	48 nM	> 50 μM	> 1042[10]	**590 ± 100 nM**	**Not detectable**	**> 10,000**
L-749329	0.6 nM	12 nM	20[11]	**One site fit 303.5 ± 34.3 nM**		**Non-selective**
Carboxylic acids						
SB209670	0.43 nM	14.7 nM	34[12]	One site fit 0.67 ± 0.14 nM		Non-selective[13]
PD156707	0.3 nM[b]	780 nM[b]	2600[14]	0.92 ± 0.38 nM	13.3 ± 2.1 μM	14,457[15]
Ambrisentan	1 nM	195 nM	195[16]	**0.28 ± 0.23 nM**	**0.25 ± 0.05 μM**	**893**
Myceric acids						
50235	81 nM	Inactive	> 1000[17]	162 ± 61 nM	171 ± 42 μM	1056[4]
S97-139	1 nM	1000 nM	1000[18]	**45.3 ± 25 nM**	**47.6 ± 9.9 μM**	**1051**

ND Not reported. New data from this study are indicated in bold to distinguish from previously published data. K_i — the equilibrium dissociation constant of a ligand determined in inhibition studies; K_D — the equilibrium dissociation constant of a ligand.
[1]Williams et al. (1993), [2]Molenaar et al. (1993), [3]Aramori et al. (1993), [4]Peter and Davenport (1996), [5]Peter and Davenport (1995), [6]Doherty et al. (1993), [7]Breu et al. (1993), [8]Clozel et al. (1994), [9]Wu et al. (1997), [10]Webb et al. (1995), [11]Walsh et al. (1994), [12]Elliott et al. (1994), [13]Johnström et al. (2004), [14]Patt et al. (1997), [15]Maguire et al. (1997), [16]Riechers et al. (1996), [17]Fujimoto et al. (1992), [18]Mihara et al. (1994).
[a] Or cells/animal tissues that endogenously express one receptor subtype exclusively or predominantly.
[b] IC_{50} value.

Table 2
Summary of ET_A receptor affinities reported for endothelin antagonists determined in binding and functional assays in human coronary artery and saphenous vein.

Antagonist	Vascular preparation	Binding	Functional	
		ET_A K_D	ET_A K_B[a]	K_B/K_D
Peptides				
BQ123	Saphenous vein	0.55 ± 0.17 nM[1]	141 nM[2]	256
	Coronary artery	0.85 ± 0.03 nM[1]	91 nM[2]	107
FR139317	Saphenous vein	**0.56 ± 0.01 nM**	87 nM[2]	**156**
	Coronary artery	0.41 ± 0.13 nM[3]	126 nM[2]	307
PD151242	Coronary artery	0.51 ± 0.07 nM[4]	1.1 μM[4]	2157
Sulphonamides				
Ro-462005	Saphenous vein	**0.15 ± 0.01 μM**	**1.4 μM**	**9**
	Coronary artery	0.19 ± 0.04 μM[3]	**2.4 μM**	**12**
Bosentan	Saphenous vein	**32.2 ± 3.2 nM**	**1.6 μM**	**50**
	Coronary artery	2.94 ± 0.95 nM[3]	**2.9 μM**	**967**
BMS 182874	Saphenous vein	**580 ± 40 nM**	**275 nM**	**0.5**
L-749329	Saphenous vein	**66.7 ± 7.5 nM**	**6.5 nM**	**0.1**
Carboxylic acids				
SB209670	Saphenous vein	**11.2 ± 1.4 nM**	**12 nM**	**1.1**
PD156707	Saphenous vein	0.5 ± 0.13 nM[5]	2 nM[5]	4
	Coronary artery	0.15 ± 0.06 nM[5]	8 nM[5]	40
Myceric acids				
50235	Coronary artery	6.8 ± 2.9 nM[6]	1.1 μM[6]	157

New data are indicated in bold to distinguish from previously published data. ND. Not determined.
K_D — the equilibrium dissociation constant of a ligand determined by means of a radioligand binding assay.
K_B — the equilibrium dissociation constant of a competitive antagonist determined by means of a functional assay.
K_B/K_D — the ratio of the equilibrium dissociation constant determined for a ligand in functional compared to that obtained in radioligand binding experiments in the same tissue.
[1]Davenport et al. (1995), [2]Maguire and Davenport (1995), [3]Bacon and Davenport (1996), [4]Davenport et al. (1994), [5]Maguire et al. (1997), [6]Maguire et al. (1994).
[a] K_B derived from Schild data with slope constrained to one or from Gaddum–Schild equation.

achieve sufficient receptor occupancy *in vivo* for clinical efficacy may be much greater than predicted by *in vitro* binding assays. Whilst this is not necessarily a problem for compounds that are either non-selective or have a very marked ET_A selectivity it may mean that those compounds that have a more marginal ET_A selectivity will have to be administered at doses at which ET_B occupancy becomes apparent and so these compounds will not behave as selective ET_A antagonists at clinically effective doses. The reason for the discrepancy in affinities determined in the binding and functional assays remains unclear. We have previously reported that the affinity of BQ123 for the ET_A receptor determined in competition binding assays using either [^{125}I]ET-1 or [^{125}I]sarafotoxin 6b (S6b) in human saphenous vein homogenates was identical but the affinity of BQ123 for ET_A receptors determined in vasoconstrictor studies in isolated saphenous vein depended on the agonist used (Maguire et al., 1996). For S6b there was little discrepancy between K_D and K_B whereas the affinity of BQ123 in the functional assay was 250 times lower than predicted from the binding assay. This observation of agonist dependence of antagonist affinity in functional assays has been well documented, at least for some endothelin receptor antagonists (see for example Hay and Luttmann, 1997) suggesting that at least some of these compounds may be negative allosteric modulators of endothelin receptors rather than neutral competitive antagonists. For the most part endothelin antagonists are not routinely screened for activity against a number of agonists in binding and particularly functional assays and therefore more data are required to determine whether ligand dependence is common for the currently available structural classes of endothelin antagonist.

Conclusions

The differential distribution and function of ET receptor sub-types (Davenport and Russell, 2001) provide the rationale for using two distinct pharmacological strategies, mixed or ET_A selective antagonism. Our study indicates that whilst antagonists reported to be ET_A

selective in cloned human receptor systems generally show an even greater degree of selectivity for ET_A receptors in human tissues, for some compound concentrations required to block ET-1 mediated functional responses, such as vasoconstriction, are much greater than predicted by binding experiments. Therefore, in order to test the hypothesis of whether selective ET_A, rather than mixed receptor blockade is sufficient for clinical benefit, antagonists such as ambrisentan and sitaxentan which display at least 1000 fold ET_A selectivity may be required to address this question in *in vivo* investigations.

Conflict of interest statement

None.

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